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PRINCIPAL INVESTIGATOR: Robert A Gross, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester NY 14642

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Robert A Gross, M.D., Ph.D.

E-Mail: robert_gross@urmc.rochester.edu

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14. ABSTRACT
Patients undergoing chemotherapy can experience a decline in cognitive abilities. While well described from a clinical perspective, little is known of the neurological substrate for this difficulty, commonly known as 'chemo brain.' We hypothesize that the cognitive difficulties experienced by patients undergoing chemotherapy are the result of impaired neurogenesis, especially in the hippocampus. We further hypothesize that agents that do not cross the blood-brain barrier will not show reduced rates of neurogenesis, in contrast to agents that readily cross into the central nervous system (CNS). Our objective is to compare the effect of drugs that enter the CNS (Cytoxan and 5-FU) with agents that do not (Adriamycin and Taxol) with respect to their ability to impair the birthing of new neurons in the hippocampus of adult mice. By testing whether chemotherapeutic agents that enter the CNS can reduce neurogenesis, we hope to develop an animal model of 'chemo brain' that will allow further studies. Furthermore, if we can show that inhibition of neurogenesis is a correlate of behavioral decline after chemotherapy, we will have provided evidence that modification of chemotherapeutic regimens – specifically, using strategies to prevent CNS entry of drugs – would be of great importance in improving the quality of life in cancer patients.

15. SUBJECT TERMS
None provided.

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INTRODUCTION:

The purpose of the work was to test the hypothesis that chemotherapeutic agents can cause a reduction in neuronal birthing in the hippocampus. The rationale for this proposition is that the hippocampus is a crucial structure for memory function; as such, any disruption to its normal functioning – including disruption of the normal process of neuronal birthing – could lessen memory function. Such a process could underlie the condition popularly known as "chemo brain," in which persons who have received chemotherapy have reduced cognitive skills. To test this hypothesis, we chose chemotherapeutic agents of two groups, those that cross the blood-brain barrier (cyclophosphamide, 5-FU) and those that do not (doxorubicin, paclitaxel); we predict, based on our hypothesis, that those that readily enter the brain would reduce neuronal birthing, whereas there would be no effect on birthing by those agents that do not enter the brain. *A limitation to this series of controls is that detailed data regarding passage through the blood-brain barrier are not available.* Mice were to be treated with these agents, and bromo-deoxy-uridine (BrdU) would be injected, thereby labeling newly birthed cells. By use of immunohistochemical techniques, we sought to determine the number of birthed neurons and their destiny – for example, differentiation into neurons or glia.

REPORT:

Extenuating Circumstances:

We had asked for and received two twelve-month extensions to continue this work, due to technical and personnel difficulties. This constitutes the final report for this study.

Report:

1) We have completed three experiments.

<u>Experiment #1: 4 mice per treatment group; 12 controls</u>

- Doses of drug administered on days 1, 4 and 7:
  - cyclophosphamide 50 mg/kg
  - 5-FU 100 mg/kg or 60 mg/kg – reduced because of ill health or death (75%) in higher dosage group
  - Doxorubicin 5 mg/kg
  - (no paclitaxel)
- BrdU administered on day 8, 4 injections q2hr 50 mg/kg
- Animals sacrificed 28 days after BrdU (to allow maturation)

<u>Experiment #2: 5 mice per group, 10 controls</u>

- Doses of drug administered on days 1, 4 and 7:
  - 5-FU 60 mg/kg
  - Paclitaxel 5 mg/kg
- BrdU administered as 4 injections q2hr on days 8-11 50 mg/kg
- Animals sacrificed 14 days after last BrdU injection

Experiment #3: 6 mice per group, 8 controls

- Doses of drug administered on days 1, 4 and 7:
  - 5-FU 60 mg/kg
  - Paclitaxel 5 mg/kg
  - Cyclophosphamide 50 mg/kg
  - Doxorubicin 5 mg/kg
- BrdU administered as 4 injections q2hr on day 8
- Animals sacrificed on day 9

We thus have experiments that allow us to assess maturation, with neuronal birthing in all cases occurring just after chemotherapy. Full maturation of birthed cells (experiment 1), partial maturation (experiment 2) and an immediate assessment of the birthing rate without maturation (experiment 3).

For all experiments, tissue was fixed *in vivo* and processed for chromagen staining with biotin-avidin. Chromagen staining was performed on every third hippocampal slice. Alternatively, fluorescent labeling of neuronal (NeuN) or glial (GFAP) markers was accomplished. In separate immunohistological analyses, we performed concurrent fluorescent labeling of BrdU and either double cortin – an early neuronal developmental marker, cytoplasmic – or cleaved caspase-3. These labelings were done as controls to assure ourselves that BrdU-labeled cells represented newly-birthed neurons and not apoptotic neurons, which are also labeled with BrdU.

2) Results:

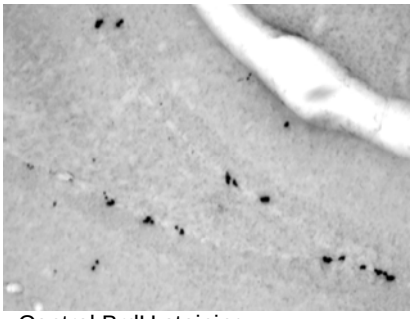
While this experiment suggested that the chemotherapeutic regimens chosen were relatively safe – in other words, that toxicity would not be a major confound – we still noted that weights declined slightly in the doxorubin- and 5-FU-treated mice, by 10-15% and 5%, respectively.

Experiments 1 and 2: After the definitive experiment (#3), it became evident that the results of these two experiments could not be used because the sectioning technique was insufficient to provide consistent cuts through the hippocampus. We have stored intact one hemisphere from each experiment, however, and will go back and section the second hippocampus to provide data on maturation of newly birthed cells. One limitation, especially for the first experiment, is that exact numbers of cells may be difficult to determine: while a 28-day period after BrdU allows time for differentiation of newly-birthed cells to neuronal or glial types, it also reduces cell counts due to cell death – those birthed cell that do not survive to a differentiated phenotype.

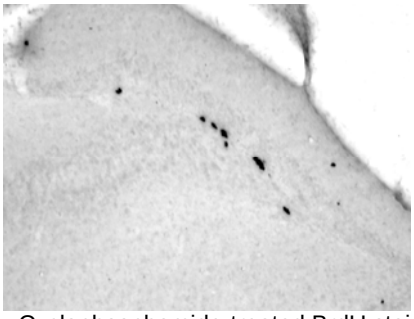
Experiment 3:

This experiment measures the effect of chemotherapeutic agents on cell birthing in the hippocampus. Sacrifice of animals the day after BrdU administration allows for comparison to Experiment 1, which followed the same drug and BrdU schedules, but allowed for maturation of cells. In the Figure, a typical control and treated hippocampal slice are shown. Immunofluorescent labeling showed the same result, with similar numbers of BrdU-labeled neurons; in this experiment, most BrdU-labeled cells are green, indicated that at this early time point after birthing, cells do not yet have a neuronal phenotype. Those that are neuronal show

co-labeling with green and red fluors, showing as yellow. As we had shown in previous work, outlined in the original proposal, newly birthed neurons develop mature markers up to four weeks after labeling, by which time the majority (~90%) have either mature neuronal (NeuN) or glial (GFAP) expression.

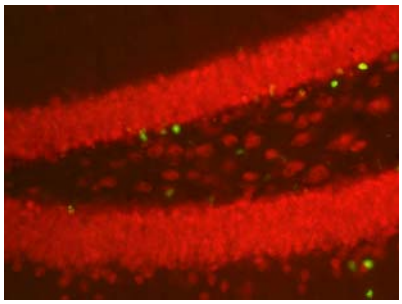


Control BrdU staining
a



Cyclophosphamide-treated BrdU staining
b

Fig 1



BrdU labeling = green; NeuN labeling = red

Fig 2

Table 1

Condition	Cells / Slice	Cells / Hippocampus (% change from Control)
	Mean ± SEM	
Controls	35 ± 1	2103 ± 56
5-FU	30 ± 1	1777 ± 57 (-16*)
Cyclophosphamide	24 ± 0.8	1462 ± 51 (-30**)
Paclitaxel	27 ± 1	1632 ± 58 (-22**)
Doxorubicin	23 ± 1	1360 ± 68 (-35**)

* $p < 0.002$
** $p < 0.001$

To assure ourselves further that BrdU-labeled cells are neuronal, we have labeled separate tissue slices from Experiment 3 with fluorescent double cortin antibodies, as double cortin is an early expression marker for neuronal differentiation. These results, shown in a typical figure below, demonstrate that many BrdU positive cells co-localize with double cortin signal (green overlapping red = yellow). However, quantifying this result is difficult, because double cortin is a cytoplasmic marker, whereas BrdU labeling – being DNA-specific – is found in the nuclear compartment. Nevertheless, we note that in neurons treated with chemotherapeutic agents, double cortin labeling is reduced, confirming the effect of chemotherapeutic agents in reducing cell (and eventually neuronal) birthing.

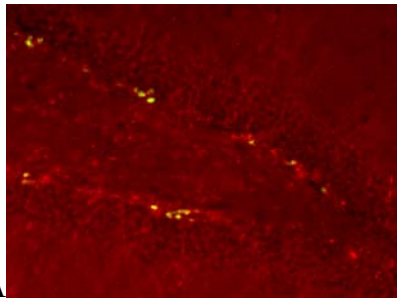
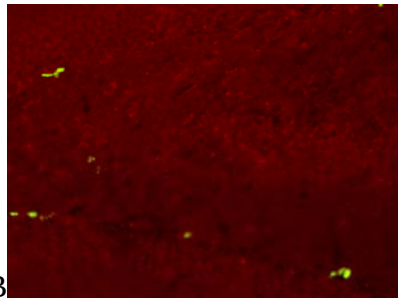
A  B 

Fig 3

Co-labeling of BrdU (green) and double cortin (red) in control (left, A) and doxorubicin-treated hippocampal slices (left, B)

Note the more robust cytoplasmic staining evident in the control slice and the reduced bright red areas indicating double cortin staining.

Having established that BrdU labeling was similar in location to double cortin staining, we performed an additional control experiment. Because BrdU can label both newly birthed cells as well as those undergoing apoptosis, and because we are relying on cytotoxic compounds to reduce neuronal birthing, we wanted to determine the proportion of neurons labeled with BrdU that were recently birthed as opposed to those undergoing apoptosis. To do this, we labeled slices with fluorescent BrdU antibodies (green) and with fluorescent antibodies to cleaved caspase-3, a marker of apoptosis. In the figure below, we show that, of BrdU-labeled cells from Experiment 3, a minority ($\leq$10%; quantification is proceeding as of the time of this report), co-label with caspase, suggesting that the majority of BrdU-labeled cells are newly birthed, and are destined for either neuronal or glial maturation. We assume that the baseline level of caspase positivity represents the number of birthed cells that do not survive; this number is likely greater than the number of BrdU-positive cells, which represent only those cells birthed during a 24-hour window.

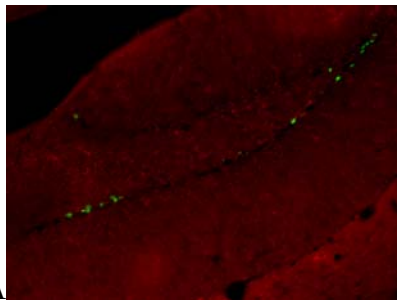
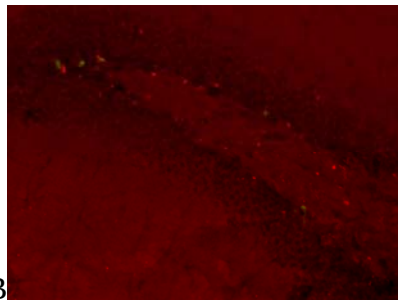
A  B 

Fig 4

Control (left, A) and doxorubicin-treated (left, B) slices, labeled for BrdU (green) and cleaved caspase-3 (red). There is an apparent increase in apoptotic cells, but not necessarily those that were newly-birthed (green).

One last experiment is being performed as of the time of this report (Experiment 4), designed to test the hypothesis that a reduction in neuronal birthing due to chemotherapeutic agents may be compensated for by administration of growth factors. The goal of this proof-of-concept experiment is to determine whether there may be strategies to reduce the effect of chemotherapeutic agents on neuronal birthing in humans, and thus serve as a preventative therapy for 'chemo brain.' Mice (groups of 5) will be treated with cyclophosphamide (crosses the blood-brain barrier; produced a large decrease in birthing), as outlined in Experiment 3. There will be corresponding control groups that do not receive cyclophosphamide. In addition, groups of mice will receive i.p. injections of growth factor (IGF-1 and FGF-2); there will be parallel groups receiving growth factor only, cyclophosphamide only and growth factor plus doxorubicin. BrdU injections will occur immediately after the chemotherapeutic regimen – as in Experiment 3 – and animals will be sacrificed the next day. Tissue processing and immunohistochemistry will occur as in previous experiments. We will assay for BrdU labeling as well as NeuN and GFAP.

3) Interpretation:

This experiment confirms our overall hypothesis, that chemotherapeutic agents may reduce cell birthing in the hippocampus, and may thus underlie the condition known as 'chemo brain.'

Our original design of the experiment was such that we included agents thought to cross the blood-brain barrier (5-FU, cyclophosphamide) and those thought not to cross (paclitaxel and doxorubicin). The idea was to have an internal control consisting of agents that ought not to affect neuronal birthing by virtue of being unable to cross into the brain. Data on chemotherapeutic agents crossing the blood-brain barrier are sparse, however, and we made assumptions based on human experience, as mice data were not definitive. Our result thus suggests either 1) that agents that penetrate the CNS poorly in humans may still penetrate sufficiently in mice to affect hippocampal cell birthing; or 2) that the agents that penetrate the CNS poorly may have secondarily disrupted the blood-brain barrier, for example by affecting overall health. In this context it is worth noting that the drugs that affected health (as measured by weight) most, 5-FU and doxorubicin, had disparate effects on birthing, being the most and least suppressive.

Having worked out the technical details, and having shown a robust significant reduction of cell birthing resulting from all four chemotherapeutic regimens, a number of questions still remain unanswered. Experiment 3 shows that cell birthing is reduced, but does not speak to the eventual fate of those cells. To address this, we plan to section and stain tissue from Experiments 1 and 2, so as to determine the cell fates, triple-labeling for BrdU, NeuN and GFAP. If our previous results are a reliable guide (references), we expect that cell maturation will have begun by 2 weeks after labeling (Experiment 2) and that it will have nearly completed by 4 weeks (Experiment 1). The proportion of BrdU-positive cells that are neuronal, glial or neither will be tabulated and, for each chemotherapeutic regimen, compared to control. We expect to similar proportions of new cells evolving to neuronal or glial lineages, although it is possible that inhibition of cell birthing could skew the development toward a specific cell type.

Future directions: Should we demonstrate that administration of growth factors reverses the reduction in cell birthing caused by chemotherapeutic agents, we will pursue experiments designed to test the dose and timing of growth factor administration for maximal benefit. Further, we hope to compare cognitive or learning abilities – using behavioral paradigms – of mice treated with chemotherapeutic agents compared to those not receiving such treatment. If reduction of neuronal birthing is reduced by administration of growth factor, we will test, in mice so treated, whether the increased neuronal numbers correlate with a change in learning ability.

REPORTABLE OUTCOMES:

No publications or grant applications to date. Once the growth factor experiment is completed, we anticipate one or two publications from our data.

CONCLUSIONS:

Our results to date are sufficient to address our hypothesis, that chemotherapeutic agents may cause "chemo brain" because of an effect on cell birthing in the hippocampus. We have yet to complete the experiments that will determine newly-birthed cell differentiation and are performing a proof-of-concept experiment, that reduction of neuronal birthing by chemotherapeutic agents is reversible by administration of growth factors. Such a strategy may allow adequate systemic treatment, but without the psychological-behavioral consequences

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